General and Comparative Endocrinology 213 (2015) 9–15

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Interferences of an environmental pollutant with estrogen-like action in the male reproductive system of the terrestrial vertebrate *Podarcis sicula*

Mariailaria Verderame *, Ermelinda Limatola

Department of Biology, University of Naples "Federico II", via Mezzocannone 8, 80134 Naples, Italy

ARTICLE INFO

Article history:
Received 1 August 2014
Revised 27 January 2015
Accepted 31 January 2015
Available online 10 February 2015

Keywords:
Nonylphenol
Testis
Epididymis
Steroid receptors
Reptiles

ABSTRACT

Nonylphenol (NP) is classified among the endocrine disruptor chemicals with estrogen-like properties. It is widely used in many industries and to dilute pesticides in agriculture, and is known to affect the reproductive system of many aquatic and semi-aquatic organisms. This study aimed to verify how NP, administered via food and water, may interfere with the reproductive cycle of a terrestrial vertebrate. Our model was the male Italian wall lizard *Podarcis sicula*, a seasonal breeding species that may be naturally exposed to environmental pollution. From our findings it emerges that an NP-polluted diet administered during the mating period causes in this lizard a slowdown of spermatogenesis and affects the testicular and epididymal structure, making it similar to that of the non-reproductive period. The distribution in the testis and epididymis of mRNA for steroid hormone receptors, i.e., estrogen α and β and androgen receptors, was also investigated. NP treatment inhibits the expression of AR, ERα, and ERβ-mRNA in spermatogonia and primary spermatocytes and causes a switch-off of the secretory activity of the epididymal *corpus* by inducing the expression of ERα.

1. Introduction

Nonylphenol (NP) is a xenobiotic compound belonging to the class of alkylphenols which are persistent and non-biodegradable environmental contaminants. NP is classified among the endocrine disruptor chemicals (EDCs) as a xeno-estrogenic substance. Due to its molecular similarity with estradiol-17β (E_2), NP mimics E_2 action, also competing for the ligand binding site of the estrogen receptors (ERs) (White et al., 1994; Lee and Lee, 1996) even if its estrogenic potency, both *in vivo* and *in vitro* systems, is less than natural estrogens (~10,000 fold lower than estradiol) (Soto et al., 1995; Jobling et al., 1996; Blom et al., 1998; Sohoni and Sumpter, 1998; Laws et al., 2000; Arukwe et al., 2001; Thorpe et al., 2001).

NP is widely used in the cosmetic, textile and leather processing industries (Jobling and Sumpter, 1993; Ying et al., 2002; Vazquez-Duhalt et al., 2005), and in agriculture as a co-formulant of pesticides or emulsifier for teat dips; it accumulates in rivers, soil, groundwater, sediment, atmosphere, sewage sludge, drinking water (Duan et al., 2014; Jin et al., 2014; Liu et al., 2014; Zhang et al., 2014), and through the food chain in organisms (Guenther et al., 2002; Muncke, 2009). In the European Union the use of NP has been limited to 0.1% in open systems (EU: Directive 2003/53/EC of the European Parliament, 2003). From a recent report by Greenpeace (January 2014) it emerged that nonylphenol ethoxylates (NPEs; up to 17,000 mg/kg) were found in many children's clothing and footwear made also by the major known brands; the washing of these clothes releases NPEs into the public wastewater systems of the countries where these products are sold.

EDCs with estrogen-like action are known to affect the male reproductive system (Carlsen et al., 1992; Milnes et al., 2006; Tripathi et al., 2009; Lagos-Cabre and Moreno, 2012) but the mechanisms by which NP interferes is not fully understood (Hong-Xia et al., 2013). Furthermore, the finding in male plasma of vitellogenin (VTG), a typical female estrogen-dependent protein, is considered the main biomarker of environmental pollution by xeno-estrogenic substances (Christiansen et al., 1998; Kinnberg et al., 2000a; Del Giudice et al., 2011; Verderame et al., 2011).

Most of the research in this field has been conducted on aquatic vertebrates collected or experimentally exposed to polluted environment (Ahel et al., 1993; Jobling et al., 1996; Christiansen et al., 1998; Arukwe et al., 2000a,b; Kinnberg et al., 2000a,b; Seo et al., 2006; Ruggeri et al., 2008; El-Sayed Ali et al., 2014; Staniszewska et al., 2014) while few studies have been carried out on terrestrial vertebrates (Han et al., 2004; Razia et al., 2006; Iguchi et al., 2006; De Falco et al., 2014) for whom the soil and feeding are the main routes of exposure.

* Corresponding author. Fax: +39 081 2535035.
E-mail addresses: ilaria.verderame@unina.it (M. Verderame), limatola@unina.it (E. Limatola).

http://dx.doi.org/10.1016/j.ygcen.2015.01.027
0016-6480/

The aim of the present research was to investigate the effects of NP-polluted diet (food and drink) on the morphology of testis and epididymis in the lizard *Podarcis sicula* that is widespread in Italy and feeds on insects, plants and pedofauna. This lizard is a seasonal breeding species, its mating period lasting from spring to early summer. In these months an intense spermatogenic activity takes place in the testis and the epididymis changes in relation to the passage of the spermatozoa (Angelini and Botte, 1992). Further, to clarify some aspects by which the NP could interfere with the reproductive cycle of this lizard we also ascertained the expression of androgen and estrogen receptor in these compartments.

2. Materials and methods

2.1. Animals and experimental protocol

Male *P. sicula* lizards (about 7 cm snout-vent) were captured near Naples (Campania, Italy) during the mating period (early May), randomly divided into three groups, kept in terraria and exposed to natural temperature and photoperiod. The experimental group ($n = 9$), undergoing treatment with NP (Etravon-Syngenta, Italy), was fed every other day for 2 weeks with larvae of *Tenebrio molitor* sprayed with an aqueous NP solution (0.25%); a drinking trough containing water polluted with NP (0.05%) was always available. The live mealworms were sprayed with NP outside the terraria and then transferred inside. Their ingestion by the lizards, which eat only living and mobile larvae, was observed. A control group (C1; $n = 5$) was fed with non-polluted food and received fresh water for 2 weeks; another untreated control group (C2; $n = 5$) was sacrificed at time 0, 2 days after capture.

All the animals were killed by decapitation after anesthesia in ice, and the testes with the attached epididymis were immediately excised and processed for histological methods. We were authorized to capture the animals for experimental treatments by the Italian Ministry of the Environment (auth. SCN/2D/2000/9213).

2.2. Histology

The specimens were fixed in Bouin's fluid (Mazzi, 1977), alcohol dehydrated and paraffin embedded. Sections 7 mm in thickness were obtained with Reichert–Jung 2030 microtome. Some sections were stained with Mallory's trichrome modified by Galgano (Mazzi, 1977); others were processed by *in situ* hybridization (ISH) and TUNEL test. All the histological results were examined using a Nikon-MicroPhot-FXA. To evaluate the size of Sertoli cells the microscope was equipped with a micrometer ocular (Leica). Student's *t*-test was used to determine which values significantly differed from controls; one-way analysis of variance (ANOVA) was used (GraphPad Prism 5 software), and significance of tests was accepted at $p < 0.05$.

2.3. In situ hybridization (ISH)

ISH was performed on adjacent sections with homologous AR, ERα or ERβ probes (Verderame et al., 2011,2012; Verderame, 2014). Briefly, the dewaxed sections were treated with proteinase K (10 μg/ml) at 50 °C for 10 min. Digoxigenin (DIG)-labeled probes were used at a concentration of 80 ng/100 μl in hybridization buffer (Tris–HCl 0.02 M, pH 7.5; NaCl 0.3 M; EDTA 0.01 M; DTT 0.1 M; Formamide 50%; Denhardt's 1×; tRNA 100 μg/ml; ss-DNA 100 μg/ml) overnight at 50 °C in a moist chamber. The slides were incubated with RNase mix at 37 °C for 30 min and in the same mix without RNase at 37 °C for 30 min, washed in 2× SSC for 3 min, in 0.1× SSC at 60 °C 15 min, and in NTP (Tris–HCl 0.1 M, pH 7.5; NaCl 0.15 M) and then incubated in 2% blocking solution (Roche Diagnostics, Mannheim, Germany) in maleic acid buffer (0.1 M maleic acid; 0.15 M NaCl, pH 7.5) for 1 h. The sections were kept overnight at 4 °C with an alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) (1:2500) in blocking solution and rinsed in NTP buffer for 30 min and in NTM buffer (Tris–HCl 100 mM, pH 9.5; MgCl 50 mM; NaCl 100 mM) for 30 min. Finally the sections were kept in the color detection substrate solution BCIP/NBT (nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate) in the dark at RT as recommended by the manufacturer (Roche) in NTM until appearance of the color.

2.4. TUNEL test

The TUNEL test was performed with the TdT-FragELTM DNA Fragmentation Detection Kit as recommended by the manufacturer (Calbiochem, Germany). Briefly, the sections were rinsed with Tris-buffered saline (TBS; 20 mM Tris, pH 7.6; 140 mM NaCl) and treated with proteinase K (20 μg/ml) for 20 min. After a wash in Tris (10 mM, pH 8), the sections were incubated with 3% H_2O_2 for 4 min to inactivate the endogenous peroxidases. The slides were incubated for 5 min with TdT buffer (200 mM Na-cacodylate; 30 mM Tris; 0.3 mg/ml BSA, and 0.75 mM $CoCl_2$, pH 6.6) and then covered with 60 μl of TdT and biotinylated dUTP (3 μl TdT enzyme and 57 μl Labeling Reaction mix) and kept for 1.5 h at 37 °C in a moist chamber. Negative controls were obtained with biotinylated dUTP in TdT buffer without TdT enzyme. The reaction was stopped by incubating the slides with stop solution (0.5 M EDTA, pH 8.0) for 5 min at room temperature. The sections were blocked in 4% BSA and then incubated with conjugated streptavidin–horseradish peroxidase for 30 min. The reactions were finally revealed with 3,3-diaminobenzidine (DAB) solution and counterstained with methyl green.

3. Results

Both the untreated animals killed at time 0 (C2) and males that received a non-polluted diet for 2 weeks (C1) showed the same features. Hence from now on they will be indicated as control males.

3.1. Testis: Histology and mRNA distribution of AR and ERs

The testes of the untreated control males stained with Mallory's trichrome showed the seminiferous epithelium filled with all germ cells from spermatogonia (spg) to spermatozoa (spz) as expected in the mating period (Fig. 1a). In the animals exposed to the NP-polluted diet the lumen of the tubules was wide (Fig. 1b), the seminiferous epithelium was reduced in thickness and several empty spaces were evident (Fig. 1b), likely due to a decrease in the amount of the germ cells even if all stages of their differentiation were detectable. Among the germ cells many spermatids (spd) were positive to the TUNEL test (Fig. 1c) unlike the untreated control animals (Fig. 1d). Sertoli cells appeared hypertrophic and enlarged (Fig. 1e and f); statistical analysis performed by measuring the diameter of 30 Sertoli cells reveals a size of about 5.14 ± 0.53 μm in control samples and of 10.74 ± 0.54 μm in NP-treated males.

In the control males the expression of ERα (Fig. 2a), ERβ (Fig. 2b) and AR (Fig. 2c) in the seminiferous epithelium occurs in all germ cells from spg to spz. In the NP-treated samples the spg and primary spermatocytes (spcI) lacked the ERα, ERβ or AR mRNA transcripts whereas they were present in the secondary spermatocytes (spcII), spd and spz (Fig. 2d–f).